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THE NITROGENASE IN A METHANOGENIC ARCHAEABACTERIUM AND
ITS REGULATION(U) NEW YORK STATE COLL OF AGRICULTURE
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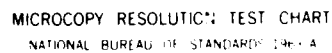
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Form Approved
OMB No 0704-0188

1a. REPORT			1b. RESTRICTIVE MARKINGS N A		
2a. SECURITY CLASSIFICATION AUTHORITY N A			3. DISTRIBUTION/AVAILABILITY OF REPORT Distribution Unlimited		
2b. DECLASSIFICATION/DOWNGRADING SCHEDULE N A			5. MONITORING ORGANIZATION REPORT NUMBER(S) N A		
4. PERFORMING ORGANIZATION REPORT NUMBER(S) O&D			6a. NAME OF PERFORMING ORGANIZATION Cornell University		
6b. OFFICE SYMBOL (If applicable) N A			7a. NAME OF MONITORING ORGANIZATION Office of Naval Research		
6c. ADDRESS (City, State, and ZIP Code) Dept of Microbiology, Stocking Hall Ithaca, NY 14853			7b. ADDRESS (City, State, and ZIP Code) 800 N. Quincy St. Arlington, VA 22217-5000		
8a. NAME OF FUNDING/SPONSORING ORGANIZATION Office of Naval Research			8b. OFFICE SYMBOL (If applicable) O N R		
8c. ADDRESS (City, State, and ZIP Code) 800 N. Quincy Street Arlington, VA 22217-5000			9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER N00014-86-K0781		
11. TITLE (Include Security Classification) The Nitrogenase in a Methanogenic Archaeobacterium and its Regulation.			10. SOURCE OF FUNDING NUMBERS		
			PROGRAM ELEMENT NO.	PROJECT NO.	TASK NO. NR441d009
12. PERSONAL AUTHOR(S) Zinder, Stephen H.			15. PAGE COUNT 5		
13a. TYPE OF REPORT Annual			13b. TIME COVERED FROM 9/86 TO 8/87		
14. DATE OF REPORT (Year, Month, Day) August 31, 1987			16. SUPPLEMENTARY NOTATION		
17. COSATI CODES			18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)		
FIELD	GROUP	SUB-GROUP	Archaeobacteria, nitrogen fixation, regulation, nutrition		
19. ABSTRACT (Continue on reverse if necessary and identify by block number)					
<p>The objectives of this project are to gain an understanding of the process of nitrogen fixation in the methanogenic archaeobacterium, <i>Methanosarcina barkeri</i> strain 227. Initial studies centered on the growth physiology of <i>M. barkeri</i> under diazotrophic conditions. We have also demonstrated that crude extracts of diazotrophic <i>M. barkeri</i> reduce acetylene at a very low rate, but apparently reduce dinitrogen to ammonia at a 20-fold higher rate per electron. Among the objectives for the coming year are further characterization of reactions carried out by the nitrogenase in crude extracts, and purification of the nitrogenase enzymes.</p>					
20. DISTRIBUTION/AVAILABILITY OF ABSTRACT <input checked="" type="checkbox"/> UNCLASSIFIED/UNLIMITED <input type="checkbox"/> SAME AS RPT. <input type="checkbox"/> DTIC USERS			21. ABSTRACT SECURITY CLASSIFICATION U		
22a. NAME OF RESPONSIBLE INDIVIDUAL Donna Garrett			22b. TELEPHONE (Include Area Code) 607/255-0414		22c. OFFICE SYMBOL O N R

PROGRESS REPORT ON CONTRACT N00014-86-K-0781

R&T CODE: 441d009--02

PRINCIPAL INVESTIGATOR: Stephen H. Zinder

CONTRACTOR: Cornell University

CONTRACT TITLE: The Nitrogenase in a Methanogenic Archaeobacterium and its Regulation.

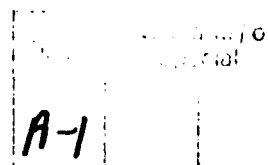
START DATE: 1 September 1986

RESEARCH OBJECTIVE: To characterize nitrogen fixation by *Methanosarcina barkeri* strain 227 and its regulation, and to purify the nitrogenase.

PROGRESS: The initial phases of the project included studying the growth physiology of diazotrophy by *Methanosarcina barkeri* strain 227 and demonstration and characterization of the properties of the nitrogenase system in crude cell-free extracts. We have completed most of this work, and a manuscript will be ready for submission after slight revision and the completion of a few minor control experiments.

Among the highlights are that molybdate at levels as low as 10 nM stimulated diazotrophic growth while tungstate inhibited it, consistent with the nitrogenase being a molybdo-enzyme. No evidence was obtained for growth stimulation by vanadium or chromium. Whole cells reduced acetylene, but at an extremely low rate, ca. $1 \text{ nmol min}^{-1} \text{ mg}^{-1}$ protein, two to three orders of magnitude lower than found in typical diazotrophs. We showed that activity was only found in dinitrogen-grown cells, and that addition of ammonia or glutamine caused a switchoff of the enzyme. Ludden's laboratory has shown that the ammonia switchoff in photosynthetic eubacteria is caused by ADP-ribosylation, and if this is the case for strain 227, it will be the first example of control of enzyme activity by modification in an archaeobacterium.

Since acetylene is a potent inhibitor of methanogenesis in strain 227 and other methanogens, we had to use acetylene at low partial pressures in whole-cell incubations, and even these low levels were somewhat inhibitory to methanogenesis. We hypothesized that crude extracts should show much higher activity since ATP and reducing power would be directly supplied to the enzymes.



However, the activity in crude extracts was similar to that in whole cells, while a control culture of *Klebsiella* showed characteristically high activity indicating that our procedures were correct. One explanation for the low activity is that acetylene is a poor substrate for the nitrogenase compared with dinitrogen. This would be in marked contrast with conventional nitrogenases which reduce acetylene and dinitrogen at similar rates per electron, but does resemble alternative nitrogenases. We have assayed ammonia production from dinitrogen by crude extracts, and have found rates over 20-fold higher per electron than acetylene reduction rates, in agreement with this hypothesis.

SDS polyacrylamide gel electrophoresis revealed a band in dinitrogen-grown cells not found in ammonia grown cells which had a much lower molecular weight (26 kdal) than the conventional iron protein (35 kdal). Another band was seen at 55 kdal. A third band near 60 kdal would be expected but may be obscured by other bands. That these proteins are not produced in ammonia grown cells is strong evidence of repression.

We have initiated the next phase of this research, the purification of the enzyme. A clear need was to have enough cell material to work with. Yields of methanol grown cells were low when grown under strictly diazotrophic conditions in mineral medium, and our original idea which was presented in the proposal was to add a limiting amount of ammonia to the medium. This did hasten growth, but did not greatly increase overall yield. A solution to this problem was arrived at when we added 0.5 g/l Difco yeast extract to the growth medium. This allowed rapid initial growth, and by the time the cells had produced 50 mmoles/liter CH_4 , they actively reduced acetylene. Presumably, the available nitrogen in the medium was depleted by this time. Using this medium, we were able to harvest ca. 30 g wet weight of cells from a 10 liter fermentor after 8-10 days of growth. This should provide us with adequate supplies of cells for working out our purification protocols.

We also have developed a practical anaerobic cell harvesting procedure. We were planning on using a New Brunswick Megaflow concentrator, but we have found that its capacity was not really great enough for a 10 liter fermentor, since appreciable clogging occurred after about three liters were concentrated. We have since purchased a MiniKros hollow fiber filtration apparatus and used it with success. This unit easily fits inside our glove-box, and since it has over one square foot of filtration area, easily meets our filtration needs.

We have received and set up our FPLC system. We purchased three columns for purification of the nitrogenase proteins: a Mono Q column, a Superose 6 column, and a phenyl Superose column. We anticipate that the Mono Q ion exchange column will be the most important, since nitrogenases are typically purified using ion exchange chromatography, and because of the enormous resolution possible with this column. We have seen over 40 peaks derived from our cell extracts, resolution comparable to gel electrophoresis.

For preliminary work, we have been operating the FPLC outside the anaerobic glove box in order to familiarize ourselves with its operation and to troubleshoot any problems that might occur. We anticipate installing the FPLC inside the glove-box within the month, but have already obtained some promising results using it aerobically. Figure 1 presents FPLC runs on the Mono Q column of crude extracts of ammonia-grown and dinitrogen-grown cells. Two major peaks are visible in the run from N_2 -grown cells which were not present in ammonia-grown cells, one designated I eluted at 0.27 M NaCl, and the other, denoted II, eluted at 0.53 M NaCl. These peaks elute at NaCl molarities similar to the Mo-Fe and the Fe proteins of eubacterial nitrogenase respectively, and are good candidates for nitrogenase proteins in strain 227. A first strategy will be to recombine these peaks after they are chromatographed anaerobically and assay them for activity. Obviously, if these peaks do not show activity, we will resort to usual strategies of pooling fractions and assaying activity to find the appropriate fractions. It has become obvious to us that we need a larger Mono Q column, since the capacity of this column is only 10-20 mg protein, and therefore we plan to purchase a column with eightfold greater capacity.

WORK PLAN (Year 2): We are essentially finished with the characterization of the growth physiology of strain 227 and are just tying up a few loose ends. We are presently trying to obtain clearer results concerning the potential role of vanadium, and we also will follow up an observation we made that after prolonged incubation in the presence of tungstate, a culture of 227 did grow under diazotrophic conditions. We intend to repeat this, and if we do get growth, we will isolate a clone and characterize this mutant. It is possible that an alternative nitrogenase is expressed in this clone.

We have some other experiments planned for crude extracts. Among them are using acid-treated extracts of strain 227 to complement nitrogenase activity in *Azotobacter vinelandii* strain UW 45 and strain CA 30 obtained from Dr. Paul Bishop. These strains are deficient in molybdenum

cofactor synthesis, and acetylene reducing activity can be restored to crude extracts by acid extracts of molybdenum-nitrogenase containing cells. We were also planning on obtaining ESR spectra of diazotrophic cells. However, we have since learned that the ESR facility at Cornell does not operate at liquid helium temperatures necessary for detection of the nitrogenase signals, and therefore we will not do these studies until we have purified enzyme, at which time it would be worth traveling to a facility which does work at liquid helium temperatures.

Clearly, our main goal for the coming year will be to purify the enzymes. We have already outlined our strategy. The FPLC system gives us excellent resolution and rapid separations, and this should help us greatly in this process.

PUBLICATIONS AND REPORTS (Year 1):

Abstracts:

Lobo, A. L., and S. H. Zinder. 1987. Nitrogenase activity in the archaeobacterium *Methanosarcina barkeri* strain 227. Abst. 87 Ann. Mtg. Amer. Soc. Microbiol.

Presentations:

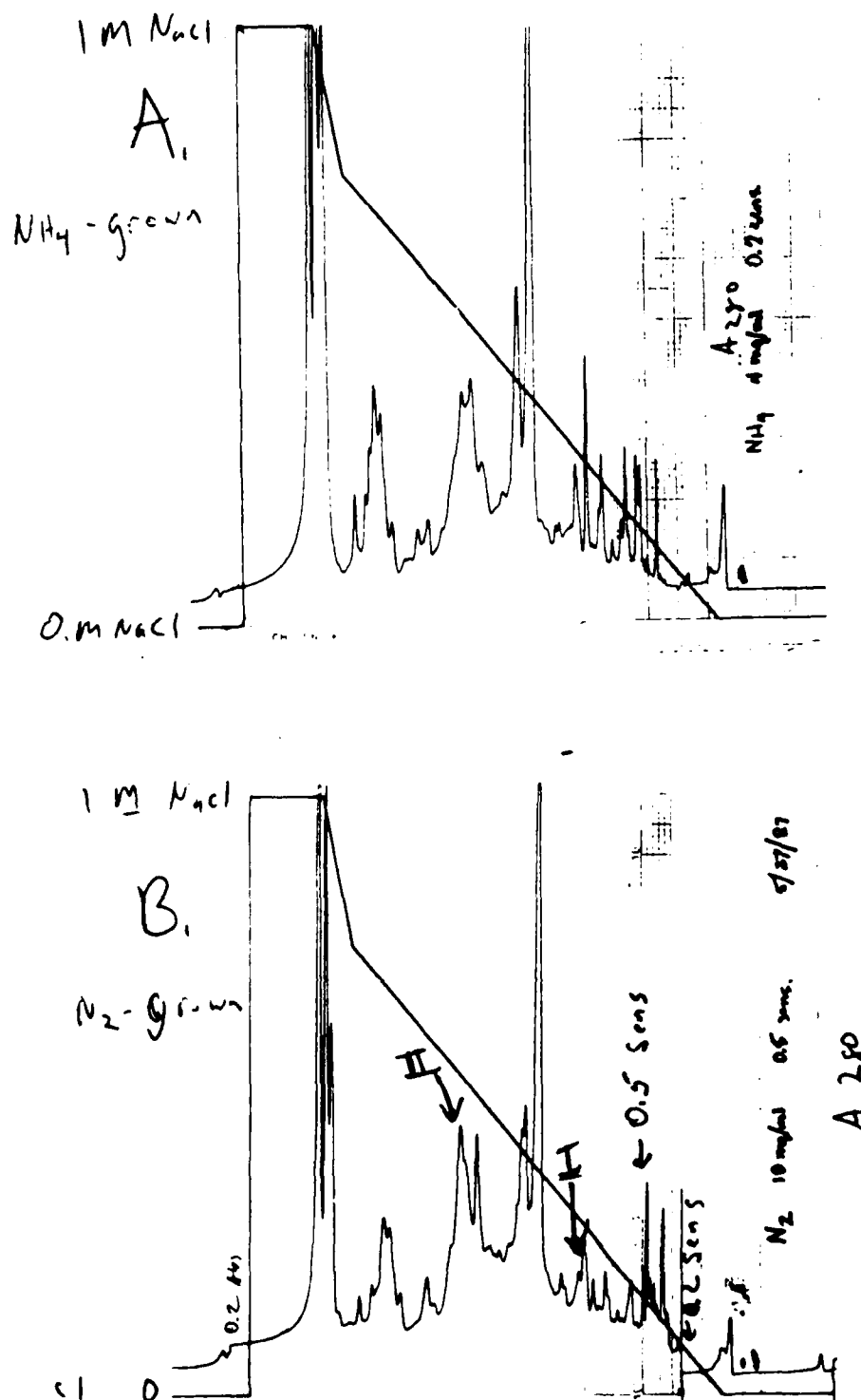
Lobo, A. L., and S. H. Zinder. 1987. Nitrogenase activity in the archaeobacterium *Methanosarcina barkeri* strain 227. Northeast Microbiology, Physiology, Ecology and Taxonomy (NEMPET) Meeting, June 8-11, 1987, Amherst, Mass.

Lobo, A. L., and S. H. Zinder. 1987. Nitrogenase activity in the archaeobacterium *Methanosarcina barkeri* strain 227. Gordon Conference on Molecular Biology of Methanogenesis, June 28-July 3, 1987.

TRAINING ACTIVITIES:

Research Assistantship for Graduate Student Anthony L. Lobo.

Figure 1. FPLC chromatograms of crude extracts from ammonia-grown (A) or dinitrogen-grown (B) cells of *Methanosarcina barkeri* strain 227. The column was a 0.5 x 5 cm Mono Q column, and the NaCl gradient ran from right to left.



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